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Running head: Brain-State Dependent Stimulation in Stroke

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Abstract:

Objective:

Adjuvant protocols devised to enhance motor recovery in subacute stroke patients have failed to show benefits with respect to classic therapeutic interventions. Here we evaluate the efficacy of a novel brain-state dependent intervention based on known mechanisms of memory and learning, that is integrated as part of the weekly rehabilitation program in subacute stroke patients.

Methods:

Twenty-four hospitalized subacute stroke patients were randomly assigned to two intervention groups; 1. The associative group received thirty pairings of a peripheral electrical nerve stimulus (ES) such that the generated afferent volley arrived precisely during the most active phase of the motor cortex as patients attempted to perform a movement; 2. In the control group the ES intensity was too low to generate a stimulation of the nerve. Functional (including the lower extremity Fugl-Meyer assessment (LE-FM; primary outcome measure)) and neurophysiological (changes in motor evoked potentials (MEPs)) assessments were performed prior to and following the intervention period.

Results:

The associative group significantly improved functional recovery with respect to the control group (median (interquartile range) LE-FM improvement: 6.5 (3.5-8.25) and 3 (0.75-3), respectively; p=0.029). Significant increases in MEP amplitude were seen following all sessions in the associative group only (p's$\leq$0.006).

Interpretation:

This is the first evidence of a clinical effect of a neuromodulatory intervention in the subacute phase of stroke. This was evident with relatively few repetitions in comparison to available techniques, making it a clinically-viable approach. The results indicate the potential of the proposed neuromodulation system in daily clinical routine for stroke rehabilitation.

Introduction

In recent years, several adjuvant therapies based on non-invasive brain stimulation (NIBS, for review see[1]) have been devised for enhancing the spontaneous biological recovery process following stroke[1,2]. The basic assumption is that NIBS 'primes' the motor cortex for subsequent learning, which then occurs during a period of increased cortical excitability. However, the benefits of NIBS on function or motor learning are relatively small[3] and the responses highly variable between and within patients. The source of this variability remains unclear, but may be related to the diffuse set of cortical neurons activated by NIBS which exhibit either inhibitory or excitatory actions onto the motor cortex[4]. The efficacy may thus be related to the overall state of excitability of the cortical network, referred to as the brain state. Applying NIBS during specific brain states may enhance their effectiveness[5].

We have recently demonstrated that a brain state-dependent peripheral stimulation protocol induces significant plasticity of the damaged cortex in chronic stroke patients that translates directly into improved function[6]. Peripheral nerve stimulation is timed to arrive at the motor cortex during the peak negative (PN) phase of the movement-related cortical potential (MRCP), inducing a causal and systematic relation between the sensory signals arising from muscles involved in the movement and the physiologically generated brain wave during imagination or attempt of that movement (Fig. 1). This intervention exhibits many of the characteristics of associative long-term potentiation, one of the primary mechanism for memory formation and learning[7,8], since its effects develop rapidly, are long lasting, depend on the timing of the two inputs and are specific to the targeted muscle[9,10,11].

One of the advantages of triggering peripheral stimulation based on the physiological activation of the motor cortex is the active participation of the patients[12]. Indeed, a very small number of pairings are sufficient to promote cortical plasticity when the delay is precisely timed[6]. This approach can be exploited in a brain-computer-interface (BCI) technology that detects the brain activation patterns of patients and triggers peripheral stimulation. Preliminary studies on healthy participants have shown the feasibility of this approach[10,11].

In the current study, we present for the first time the concept of brain-state dependent peripheral stimulation in subacute stroke patients. We hypothesized that the proposed intervention would lead to an increase in function of the affected limb that is directly measurable through clinical

scales. Further, we hypothesized an enhancement of the output of the motor cortex to the target muscle following a very short intervention time (ideally, even within a single session). The demonstration of this hypothesis would strongly support the theory that timing is critical and that associativity is the main physiological mechanism underlying the induced plasticity.

Methods

Ethical approval

Patient demographic data and baseline clinical evaluations are shown in Table 1. Eighteen male and six female patients (61.2 ± 8.2 years) participated in this study. Inclusion criteria were; age above 18 years, a superior division middle cerebral artery (MCA) stroke within four months of inclusion in the study, and the ability to follow instructions. All patients underwent neuropsychological assessment, with none meeting the DSM-IV criteria for diagnosis of dementia. Patients were excluded if they presented with concomitant neurological or other severe medical problems, seizure history, contraindications to transcranial magnetic stimulation (TMS), cognitive impairments, treatment with drugs that act on the central nervous system, cardiovascular or respiratory symptoms contraindicative of walking, and any other significant non-stroke-related impairments affecting walking. All patients were inlaid at the neurorehabilitation center at Neuroenhed Nord, Brønderslev, Regionshospital Nordjylland, Denmark where they received intensive, multidisciplinary individualized rehabilitation therapy. Participation in this study was in addition to all therapies delivered at the hospital and all hospital staff were blinded to the experimental protocol. Approval for the study was given by the Scientific Ethics Committee for Nordjylland, Denmark (reference no. N-20160016). The study was performed in accordance with the Declaration of Helsinki.

Sample size calculations were based on pilot testing of the current protocol in three sub-acute stroke patients who improved 6 ± 3 points on the lower extremity Fugl-Meyer (LE-FM) motor performance assessment. Our control group was expected to improve by 1.5 ± 2 points [13]. A power analysis revealed that the minimum sample size was n = 10 in each group necessary to achieve a statistical power of at least 95% (two-tailed $\alpha = 0.05$).

Overall study design

Patients were randomly allocated to one of two groups: an associative intervention group and a sham intervention (control) group. A posteriori validation verified that the groups matched for age ($t_{(22)}$ = 0.19, p = 0.85) and time since stroke ($t_{(22)}$ = 0.36, p = 0.73). Patients attended three intervention sessions (see 'Interventions') per week for four weeks for a total of twelve sessions. During intervention sessions one, six and 12, corticospinal output properties were assessed using TMS (see 'Quantification of corticospinal output'). Immediately prior to and after the four-week intervention period, patients were assessed with several clinical scales by a clinician blinded to the experimental protocol (see 'Clinical and behavioral measures').

Clinical and behavioral measures

Assessment were made by a clinician blinded to the protocol and included the modified Ranking scale score (mRS)[14], LE-FM motor performance assessment[15], the Ashworth scale for spasticity (ASS) of the affected leg[16] and the functional ambulation classification (FAC) scale[17] and the 10-m walk test at their fastest pace[18]. The choice of using only one trained clinician was based on previous literature that demonstrated high test-retest and interrater reliability of the mRS scale [19,20], LE-FM scale [21], the ASS scale [22] and the FAC scale [23].

Movement-related cortical potential (MRCP)

During all sessions, monopolar electroencephalographic (EEG) signals were recorded using an active EEG electrode system (g.GAMMAcap[2], gTec, GmbH, Austria) connected to a g.USBamp amplifier (gTec, GmbH, Austria) from FP1, Fz, FC1, FC2, C3, Cz, C4, CP1, CP2, and Pz according to the standard international 10-20 system. The channel selection was based on the large Laplacian, with Cz as the central channel [24]. The reference electrode was placed on the left or right earlobes and the ground electrode on Fz. A single channel surface electromyogram (EMG) was recorded from the tibialis anterior (TA) muscle of the affected leg to control for the patients' movement. All EEG and EMG signals were sampled at a frequency of 256 Hz and hardware filtered from 0 to 100 Hz.

Patients were asked to attempt 30 dorsiflexion movements of the foot contralateral to the lesion site in relation to a visual cue. The experimental setup and cue is depicted in Figure 1. A custom-made Matlab script (R2014b, Mathworks®) provided this cue via a screen positioned 1.5 m in

front of the patient on when to mentally prepare, execute, and release the movement. Patients were instructed to attempt to perform a single dorsiflexion movement as fast as possible when the cursor had reached the upwards turn and to maintain the new position for 2 s, following which they relaxed again for 4-5 s prior to the next cue being provided. Data from recorded EEG signals were used to quantify the time of PN of the MRCP's before proceeding to either the associative or sham interventions described under the section 'Interventions'.

Feature extraction from the MRCP

Matlab software (R2014b, Mathworks®) was used to filter the continuous EEG signals using a second order band-pass filter from 0.05 to 10 Hz. EEG data were then divided into 4 s epochs (from 2 s before to 2 s after the visual cue) for each movement and a Laplacian channel[24] was used to enhance the MRCP in each epoch. Next, a window of 500 ms on either side of task onset was chosen. If any epochs' PN was outside the selected window it was discarded. Epochs with electrooculography (EOG) activity exceeding 140 μV were also discarded. The remaining epochs were averaged and the mean PN was defined as the time of occurrence of the minimum value of the averaged MRCP in relation to the visual cue. The mean PN was used to calculate the point in time for when to apply the peripheral stimulation in the subsequent intervention session for both patient groups.

Recording and stimulation

EMG activity was recorded by surface Ag/AgCl electrodes (20 mm Ambu Neuroline 720, Ambu A/S, Denmark) placed over the belly of the TA muscle affected leg[25]. Surface EMGs were pre-amplified and sampled at 2 kHz using scientific software Mr. Kick II 2.3 (Knud Larsen, SMI®, Aalborg University, Denmark) for recordings of the motor evoked potentials (MEPs) evoked by TMS in the TA during sessions one, six and 12. During the intervention, EMG data were collected using the g.USBamps (gTec, GmbH, Austria) at a sampling frequency of 256 Hz.

A monophasic Magstim 200 (Magstim Company, Dyfed, UK) with a focal figure of eight double cone coil (110 mm diameter) was used to apply single TMS pulses to elicit a MEP in the TA. The direction of the current was directed from posterior to anterior. MEPs were elicited before (pre), immediately after (post), and 30 min after (post30) the cessation of the intervention for both groups during intervention sessions one, six and 12. For procedure, see the section 'Experimental procedures'.

Stimulation (pulse width 1 ms) of the CPN was applied by a NoxiTest isolated peripheral stimulator (IES 230, Aalborg, Denmark). Stimulating electrodes (32 mm, PALS! Platinum, Patented Conductive Neurostimulation Electrodes, Axelgaard Manufacturing Co., Ltd. USA) were placed on the skin overlying the deep branch of the CPN (L4 and L5) contralateral to the lesion site with the cathode proximal. A suitable position for stimulation, defined as the site where a maximal M-wave was produced in the TA with no activity from the synergistic peroneal muscles and no activity from the antagonist soleus (SOL), was identified. The stimulation site corresponded to a point just anterior to the level of the caput fibulae. Initially, the motor threshold (MT) was determined as the intensity where an M-wave became visible in the EMG signal. For the associative intervention group, the stimulation intensity was set to MT. For the sham intervention group, the stimulation intensity was set to ~70% of perception threshold.

Quantification of corticospinal output

Patients were seated comfortably with their affected foot resting on a footplate. Initially, the intensity for the magnetic stimulus was set at approximately 50% of the stimulator output (SO) to find the optimal site for evoking a MEP in the TA. Three consecutive stimuli at a 5-7s inter-stimulus interval, were delivered over Cz and this was repeated for different sites by moving the coil in ~1 cm steps anteriorly and laterally. The best spot for stimulation (also termed the hot-spot) was defined as the coordinate where the peak-to-peak amplitudes of the MEPs were greater in the target muscle than the amplitudes of adjacent coordinates for a given stimulus intensity. For all patients, this site was approximately 2–3 cm anterior to the vertex. Once the hot-spot was identified, it was marked on the patients' head with a felt pen to ensure that the coil position was maintained and the stimuli were consistently delivered over the same area of the motor cortex.

Subsequently, the resting motor threshold (RMT), defined as the highest stimulus intensity that produced no more than five of ten consecutive TA MEPs with a peak-to-peak amplitude of ~50 μV while the muscle was at rest, was identified. Next, ten MEPs were elicited in the resting TA at each of six TMS intensities; 90, 100, 110, 120, 130, and 140% of RMT (60 MEPs in total). The TMS stimuli were delivered every 5–7 s in a randomized order for intensity. The mean peak-to-peak TA MEP amplitudes were extracted pre, post, and 30 min following the cessation of the intervention.

Interventions

For the associative group, the intervention protocol consisted of a single electrical stimulation delivered to the common peroneal nerve (CPN) at MT and so that the artificially generated afferent flow arrived at the PN of the MRCP, as outlined in our previous publications[9,6]. The timing was calculated based on the following equation: mean PN – 50 ms. The 50 ms represents the mean latency for the afferent inflow resulting from the peripheral stimulus to reach the somatosensory cortex plus a cortical processing delay and is based on previous work[26]. For the sham group, the electrical stimulus was delivered at the same time as for the associative group but at an intensity below motor threshold (~70%) to ensure that no afferent inflow reached the cortex at the time of PN. A total of 30 pairings of attempted movement according to the cue (Fig 1) and ES were applied during each intervention session. Patients attended a total of twelve separate intervention sessions, with three sessions per week over four weeks. A minimum of 24 h elapsed between sessions. Patients were blinded as to the intervention they received.

Statistical analyses

The main outcome measures were the clinical tests and the changes in MEP amplitude. Any differences in pre-intervention clinical measures (mRS, LE-FM, ASS, and 10-m walking test) between groups were evaluated with Mann-Whitney U tests. To test whether there was a change in clinical measures due to the interventions, separate Wilcoxon signed ranks tests were employed for each group for the mRS, LE-FM, ASS scores, and the 10-m walking test speed. To compare improvements in these clinical measures, Mann-Whitney U tests analysed the absolute pre-post intervention differences between the associative and sham groups. 10-m walking test speed was analysed using non-parametric tests because of violations to the assumption of normality. Bonferroni adjustments were applied to correct for multiple comparisons. A two-way between-within ANOVA, with session (sessions one, six, 12) as the within-subjects factor and group (associative, sham) as the between-subjects factor, was used to evaluate RMT and the pre-measures of TA MEPs evoked at the highest stimulation intensity across testing sessions and groups. Greenhouse-Geisser corrected degrees of freedom were used to correct for violations of the assumption of sphericity. Finally, changes in TA MEP were analysed by a repeated measures mixed effects ANOVA. Subject was a random effects factor nested within group (associative, sham) with session (sessions one, six, 12), time (pre, post, and post30), and stimulation intensity

(90%, 100%, 110%, 120%, 130%, 140% RMT) as within-subjects fixed factors. Post hoc Fisher's least significant difference corrections were administered to determine the locus of the differences[27]. Differences with a probability of < 0.05 were considered significant. Statistical analyses were conducted in Minitab 18.

Results

Clinical measures

Baseline clinical scores for both groups are shown in Table 2. There were no statistically significant differences between the associative and sham groups for the LE-FM (95%CI: [-7, 3]), mRS (95%CI: [-1, 1]), FAC (95%CI: [-1, 1]), ASS (95%CI: [0, 0]), or 10-m walking speed (95%CI: [-0.65, 0.58] m/s; all p's > 0.50) upon enrollment. At baseline, a total of eight patients presented with no visible voluntary muscle activation of the TA and were unable to perform the dorsiflexion movement, thirteen patients had limited dorsiflexion abilities as quantified by the LE-FM scale, while three patients were able to perform a complete dorsiflexion movement.

Figure 2A shows the individual and median improvements in LE-FM scores following the associative or sham interventions. The absolute pre-post intervention period difference scores for both groups are shown in Figure 2B. The associative group significantly improved in their median (interquartile range, IQR) lower extremity LE-FM from 23.5 (18.75–26.25) to 32 (26.25–32), $Z = 3.06$, $p = 0.002$. The sham group also significantly improved from 25.5 (18–30) to 29.5 (21–31), $Z = 2.63$, $p = 0.009$. However, there was a significant median absolute pre-post intervention difference between the associative (6.5, 3.5–8.25) and sham (3, 0.75–3) groups, indicating that the associative group improved significantly more on the LE-FM compared to the sham group, $Z = 2.19$, $p = 0.029$, 95%CI: [1, 6].

The associative and sham groups significantly improved in their mRS scores from 4 (3-4) to 2.5 (2-3.3) and 4 (2.8-4) to 3 (2-3), respectively (both Z's ≥ 2.13, both p's ≤ 0.033), with no significant difference in improvements between groups ($p = 0.93$, 95%CI: [-1, 1]). Additionally, both groups equally improved their FAC score from 2 (1.8-4) to 4 (3.8-5) and 2 (1.8-4) and 4 (4-5), respectively (both Z's = 2.71, both p's = 0.007), with no difference in improvement ($p > 0.99$, 95%CI: [-1, 1]). For the ASS, no significant changes were detected for either the associative or sham groups (both p's > 0.30, 95%CI: [0, 0]).

Two patients from each of the associative and sham groups could not walk either at baseline or after the 4-week intervention period. There were five patients in the associative group and three patients in the sham group that could not walk at baseline but could walk after the four-week intervention period. In these instances, patients were assigned a walking speed of 0 m/s and

included in the statistical analyses. The associative and sham groups significantly improved their walking speed in the 10-m walking test from 0.59±0.77 to 1.09±0.78 m/s and 0.63±0.67 to 0.95±0.75 m/s, respectively (both Z's $\geq$ 2.67, both p's $\leq$ 0.008), with no difference in improvement between groups (p = 0.56; 95%CI: [-0.21, 0.56] m/s).

Reliability of the MRCP

The associative intervention as applied in the current study requires the MRCP to be stable within a session, since the arrival of the afferent inflow to the motor cortex has to occur at the precise time of the PN phase of the MRCP. However, since each session commenced with the identification of the occurrence of the time of PN of the MRCP, variability across days is expected and may even be a further marker for plasticity induction. The two upper panels of Figure 3 show the time of occurrence of the PN of the MRCP for each session for two patients. Also shown are the standard deviations. The lower panel displays the average PN time across all sessions for each patient in the associative group. Across all patients, the time of PN of the MRCP occurred at -60 ±55 ms prior to the cue to commence the movement.

Changes in corticospinal output properties

Because of patients' compliance to TMS, it was not always possible to elicit MEPs. For session one, 11/12 patients were included in the analyses from each group. For session six, 7/12 associative group patients and 8/12 sham group patients were included in the analyses, and for session 12, 11/12 associative group patients and 10/12 sham group patients were included. The RMT did not change before or after the training for either group. For the associative group, the mean (± SD) RMT was 56.8±16.6%, 59.0±13.3%, and 53.9±9.5% MSO in sessions 1, 6, and 12, respectively. For the sham group, the mean RMT was 49.2±12.3%, 48.1±11.2%, and 53.3±13.3% MSO in sessions 1, 6, and 12, respectively. A two-way between-within participants ANOVA revealed no significant interaction between group and session (p = 0.14), nor main effect of session (p = 0.95) or group (p = 0.23). In patient A05, the RMTs were 84 and 73% MSO for session one and six respectively and for patients A07 it was 79% MSO for session one. Thus, it was not possible to obtain a complete recruitment curve up to 140% RMT on these occasions.

The amplitude of the TA MEPs evoked at the highest stimulation intensity before the commencement of the intervention sessions across all patients attained values of 323±182 μV,

303±220 μV, and 425±224 μV for the associative group and 471±299 μV, 382±306 μV, and 454±500 μV for the sham group for sessions one, six, and 12, respectively. There was no significant session by group interaction, nor any main significant effects of session or group (all p's > 0.52), indicating that the maximal pre-intervention session MEPs did not change systematically throughout the intervention period.

Figure 4 shows the mean TA MEP amplitude for the patients in the associative (Figure 4A-C) and sham group (Figure 4D-F), plotted against TMS intensity for intervention sessions one, six, and 12. Data are expressed as a fraction of the maximum TA MEP amplitude prior to the intervention of the respective session. The linear mixed model analysis on TA MEP amplitudes revealed no significant four or three-way interactions between session, time, stimulation intensity, or group (all p's > 0.80). However, there was a significant two-way interaction between time by group, $F_{(2,964)} = 3.72$, $p = 0.024$ (Figure 4G). Following the significant group by time interaction, post hoc analyses revealed that, for the associative group, TA MEP amplitudes were significantly larger immediately post (291±214 μV) and 30 minutes post-intervention (323±275 μV) compared with pre-intervention values (243±241 μV), regardless of testing session and stimulation intensity ($p = 0.006$, 95%CI: [13, 80] μV and $p < 0.001$, 95%CI: [43, 110] μV, respectively). There was no difference between post and post30 MEP amplitudes ($p = 0.076$, 95%CI: [-3, 63] μV). For the sham group, there were no differences in TA MEP amplitudes between pre (233±232 μV), post (222±219 μV), and post30 (219±239 μV) measurements across testing sessions and stimulation intensities (pre-post: $p = 0.97$, 95%CI: [-33, 32] μV; pre-post30: $p = 0.38$, 95%CI: [-18, 47] μV; post-post30: $p = 0.36$, 95%CI: [-17, 47] μV).

Discussion

This is the first systematic study on subacute stroke patients that explicitly explores the associative long-term potentiation theory within a brain-state dependent rehabilitation approach. Patients enrolled in the associative intervention improved motor function significantly more compared with the sham group. The implications are that the intervention presented here has the potential to boost recovery beyond the spontaneous biological recovery processes in the first few months after the insult. Further, it opens the possibility to develop an online BCI system for patients where the intention to move is detected from continuous monitoring of the brain signals and used to trigger the peripheral stimulation that generates the afferent feedback to the brain at the precise time of the PN phase of the MRCP. This online BCI has been tested in healthy participants where it led to significant increases in the excitability of the cortical projections to the target muscle[10,11]. In the current study, we specifically tested the scientific hypothesis of associative plasticity without the BCI component to eliminate confounding factors (such as the fact that different patient groups may have different detection accuracy). Nonetheless, the current results have direct implications for a future BCI system that allows stroke patients to control their own recovery process.

To date, clinical studies on the use of BCI for stroke therapy have involved chronic patients. Although this choice simplifies the study design because of the stable conditions of the patients, a real impact in stroke therapy can only be achieved in the acute and subacute phase of the stroke. This is the time window critical for recovery since the greatest gains are achieved in this interval[28]. During this time, genes and proteins for synaptogenesis, neuronal growth and dendritic sprouting, are expressed to a greater extent following a stroke[29]. It is in this state that the brain is highly plastic and it is likely that the same synaptic rules for learning and memory formation will lead to the most optimal outcome[28]. Addressing patients in this time window is extremely challenging for neurotechnology developments because of the variability of symptom distribution and symptom severity[28] as well as limited patient compliance and variability in brain electrical activity. These conditions impose strong constraints in the development of the technology as proposed here since it relies on the accurate and early detection of movement intention from EEG signals. While chronic stroke patients exhibit rather stable MRCPs between days[6], this is not the case for subacute stroke patients (Figure 3). This necessitates the collection

of a training data set prior to the associative intervention where patients attempt the motor task in a number of repetitions. However, in the proposed intervention, each session only requires a maximum of 20-25 minutes which includes the preparation time. This is well within the time-frame typically used for other therapeutic procedures. In this way, our approach is easily transferred into the clinical setting.

Neural repair following stroke specifically involves adjacent sites around the lesion as well as remote sites that are connected to the damaged area[30]. Generally, the increased activity in remote sites as well as increased activity in the contralesional hemisphere during motor execution declines with recovery and the degree of this decline is correlated with the amount of function regained[31]. Within the time window of spontaneous biological recovery, significant axonal sprouting occurs that has the potential to be the target for novel therapies, although not all sprouting processes are beneficial[32]. The intent of the associative intervention as presented here is thus to guide plasticity by directly activating specific pathways known to be dysfunctional following a stroke. By repetitively pairing the intent of the patient, as quantified by the MRCP, with the artificially generated afferent inflow to the ipsilesional motor cortex, the associative intervention directly follows the principles of memory formation and learning first proposed by Hebb[33]. This targeted plasticity induction significantly improved functionality, and may thus promote beneficial plasticity processes such as axonal sprouting between those cortical areas that should be connected and between peripheral sensory receptors and cortical areas[28]. Indeed, sensory information from muscle receptors plays an integral part in motor learning[34]. The fact that the patients exposed to the associative intervention improved significantly more than the sham group supports the importance of the correct pairing in time of the peripherally generated signal and the MRCP. It should be noted that there were no significant improvements in the secondary functional outcome scores, i.e. the ASS or mRS. This is in agreement with previous reports [6,13,35]. The reasons for the lack of improvements in these functional outcome scores remain speculative but, at least in the current study it can be partly explained by the fact that only two patients presented with spasticity at enrolment.

The rationale of the proposed intervention is similar to that underlying paired associative stimulation (PAS – for review see[36]). PAS uses a peripherally generated afferent volley, as in our approach, and combines it with a second stimulation to the area of the motor cortex representing the target muscle with TMS. The inter-stimulus interval is such that the afferent volley arrives

just prior to the TMS stimulus. However, PAS is not as effective as our intervention since approximately 50% of participants do not exhibit the expected change in excitability following PAS[36]. The lower efficacy of PAS may be due to a diffuse activation by TMS of a set of both inhibitory and excitatory cortical neurons. In agreement with this interpretation, NIBS protocols that use either TMS or direct transcranial current stimulation have shown large variability in their effects, between individuals as well as within individuals across days[1]. In our approach, the activation of the cortical areas occurs naturally through the patient's own attempt at performing the movement and thus the relevant brain areas are activated in a more natural manner. However, what remains to be investigated is the exact site of plasticity induction. Thus, in the current study, the significant functional improvements as quantified by the LE-FM scale and the 10 m walk test, were accompanied by significant increases in MEP size only in the associative group. While this is an indication of plastic changes within the corticospinal tract [37,38], in future studies it will be important to identify the exact locus of these changes and thus the associated functional changes using techniques such as resting functional magnetic resonance imaging or diffusion weighted images [38].

The MRCP and the time of the PN of the MRCP varied from one session to the next for individual patients. Although this variability was reduced for single trials within a single intervention session, values still ranged between 150 and 300 ms. In the conventional PAS protocol, a difference of only 5 ms between the timing of the two stimuli could induce an inhibitory rather than a facilitatory effect[39]. Thus, although our associative intervention is modelled on PAS, it is unlikely that the two interventions have the same sites for plasticity induction. Irrespective of the exact site, one factor that is likely to contribute to the enhanced effect of our protocol as compared to PAS is that it is a behavioral training where the patient is actively involved in the intervention. The patient engagement combined with the correlated activation of the relevant brain areas through the peripherally generated volley and the MRCP lead to beneficial effects that are not seen in the sham intervention.

Since the patients investigated here were hospitalized, one important goal during the development of the associative intervention was that it had to be complementary to the other activities the patients had to perform as part of the standard rehabilitation procedures in Denmark. Typically, patients at this stage have difficulty to concentrate for long periods of time and experience more fatigue as compared to chronic stroke patients. The associative intervention

introduced here, requires only 30 movements to be performed in the initial phase where the PN of the MRCP is established, and 30 in the actual intervention phase. The total duration of a single session that includes preparation of the EEG and stimulation electrodes is 20 minutes. It thus paves the way for this novel technology to be used within the daily clinical practice.

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Author contributions:

N.MK., A.J.T.S., H.R., K.S. and D.F. contributed to the conception and design of the study. N.MK., A.J.T.S., S.A., H.S., K.S. and N.J. contributed to data acquisition and analysis; N.MK., A.J.T.S., N.J. and D.F. contributed to drafting the text and preparing the figures.

Conflicts of interest:

Nothing to report.

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Figure Legends

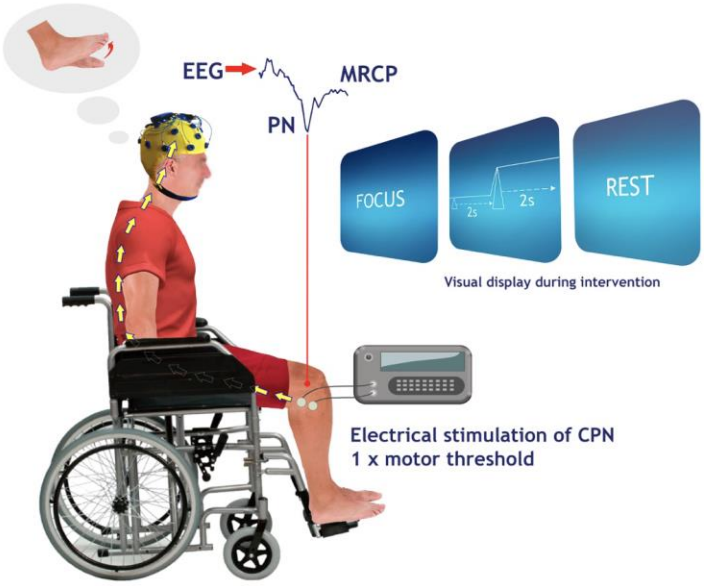
Figure 1. Schematic of the experimental set-up. Schematic of the associative and sham interventions performed during each of the 12 intervention sessions (three times per week for four weeks). Patients watched a screen placed 2 m in front of them on which a cue provided information on when to attempt the dorsiflexion movement. FOCUS appeared on the screen initially followed by the schematic of a step function. Patients were required to start the attempted movement once the moving cursor (triangle) reached the upward slope. Finally, the word REST appeared last on the screen prior to the start of the next trial. Relevant brain activity was measured, detected and the time of the peak negative (PN) phase of the MRCP extracted in the first 30 trials. In the subsequent 30 trials, this time was used to provide into an output command for an electrical stimulator. The stimulator applied a single pulse (1-ms duration) to the deep branch of the common peroneal nerve (CPN). For the associative intervention group, the induced sensory signal produced by the electrical stimulation applied to the CPN was timed to arrive at the motor cortex during the time of maximum activation of the motor cortex as seen in the electroencephalographic (EEG) signal. The stimulation intensity was set to 1 x motor threshold (MT). For the sham intervention group, the stimulation intensity was set below perception threshold such that there would be no resultant afferent volleys sent to the cortex. Thirty such pairs were performed.

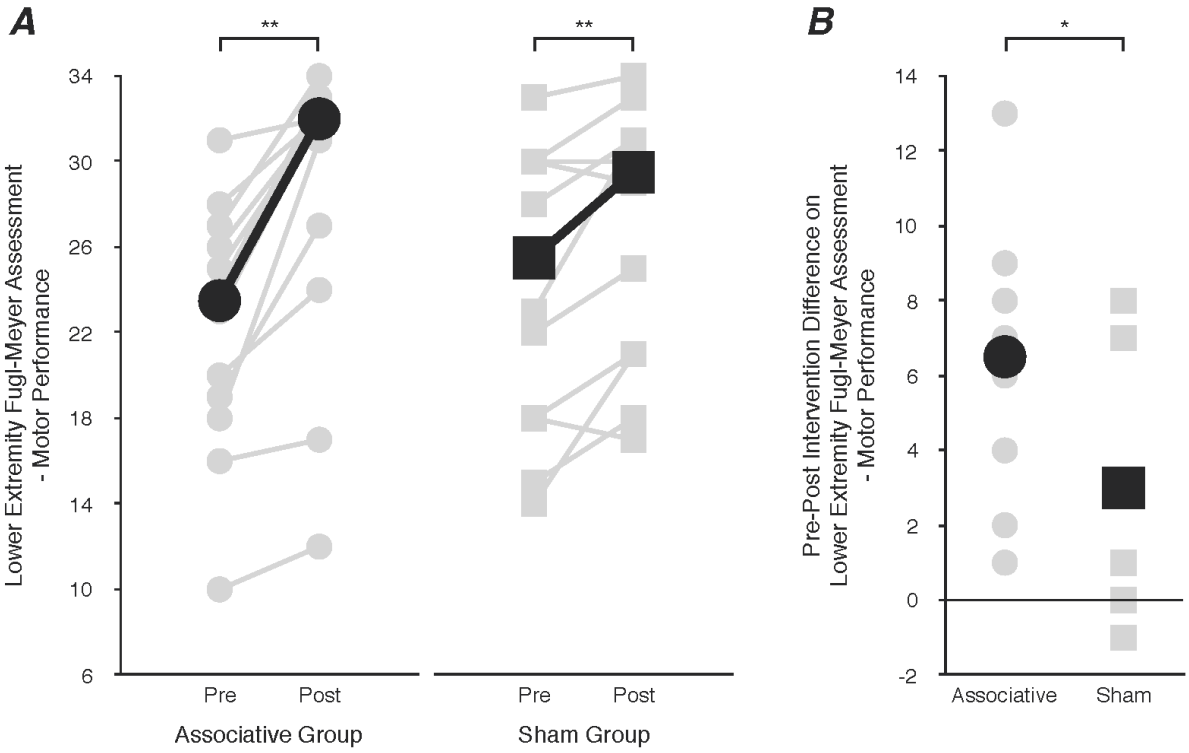
Figure 2. **Lower extremity Fugl-Meyer (LE-FM) motor performance scores improved significantly for both associative and sham intervention groups, with a greater overall improvement for the associative group. A)** LE-FM motor performance scores for all associative (grey circles) and sham (grey squares) patients pre- and post-intervention. Black circles and black squares represent median scores for the associative and sham groups, respectively. **B)** Pre-post intervention absolute difference scores for all associative (grey circles) and sham (grey squares) patients. The black circle and black square represent median absolute pre-post intervention difference scores for the associative and sham groups, respectively. *, $p < 0.05$, **; $p < 0.01$.

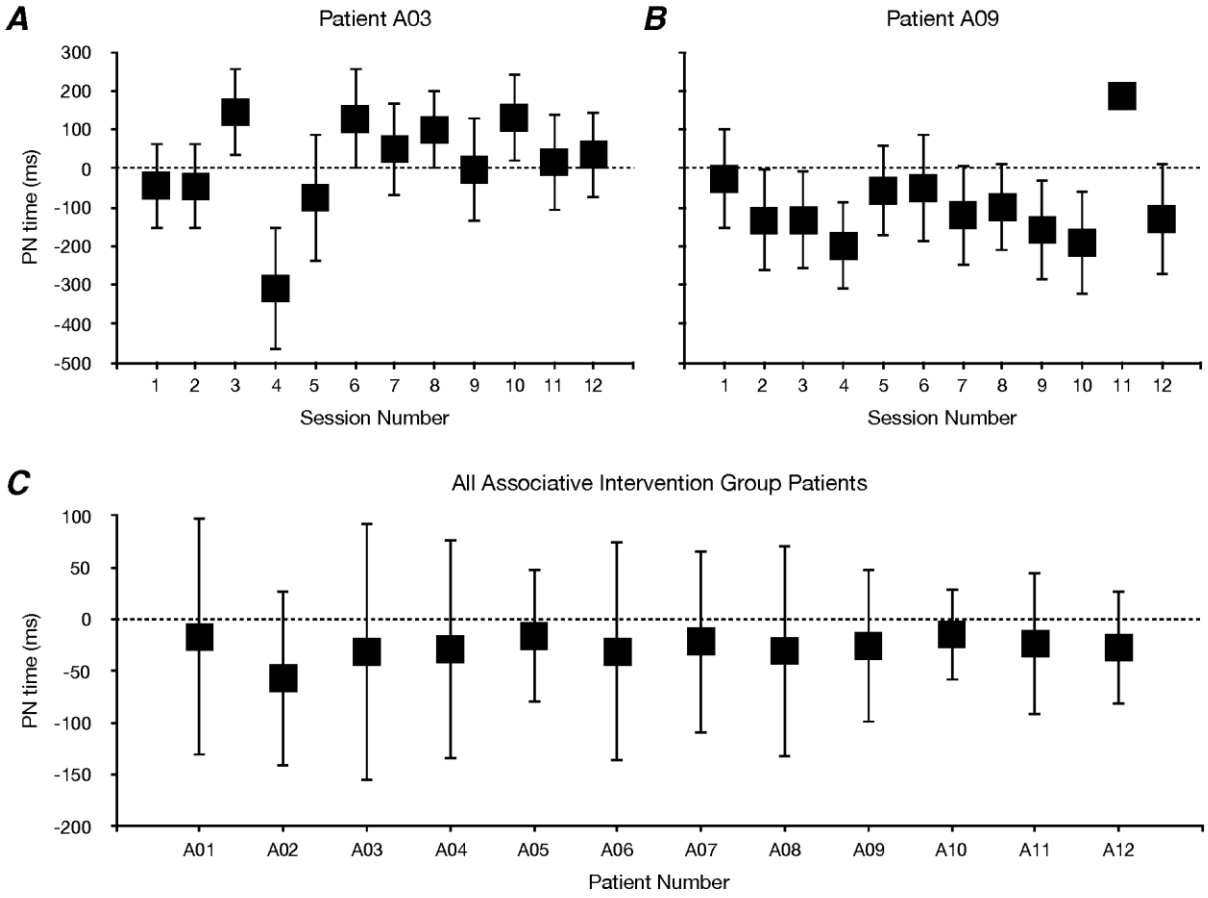
Figure 3. The time of peak negativity of the movement-related cortical potential (MRCP) across all 12 intervention sessions for the associative group. (A-B) data for single patients and each intervention session. Error bars represent standard deviations across all trials within a single

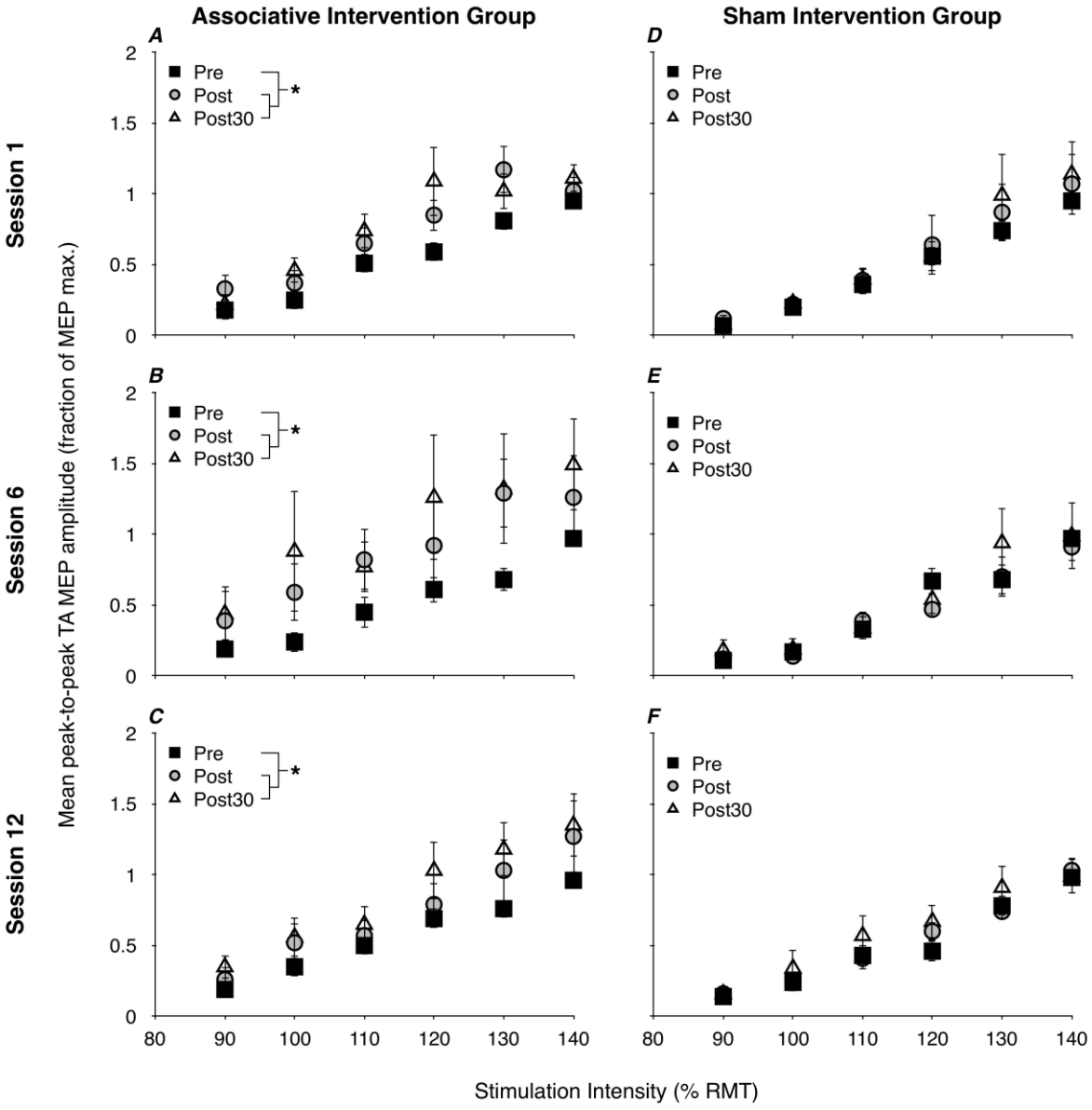
session. **(C)** Mean data across all sessions for each patient. Error bars represent standard deviations for all 12 sessions.

Figure 4. Mean tibialis anterior (TA) motor evoked potential (MEP) recruitment curves for associative and sham intervention groups for sessions one, six, and 12. Recruitment curve properties of the TA MEP before (black squares), immediately after (grey circles), and 30 minutes after (white triangles) the cessation of the associative **(A-C)** or sham **(D-F)** interventions across all participants. TA MEP amplitude is expressed as a fraction of the maximum peak-to-peak TA MEP amplitude before any intervention. Each graph represents a different day of the intervention; session one, session six (after two weeks), and session 12 (after four weeks). **G**) displays mean TA MEP amplitudes (μV) for the associative (black bars) and sham (grey bars) intervention groups for pre, post, and post30 measurements, pooled across all sessions and stimulation intensities. Note the significant group by time interaction, where MEPs are significantly increased at post and post30 measurements for the associative intervention group only. Error bars represent standard error and asterisks indicate significant differences. RMT, resting motor threshold.









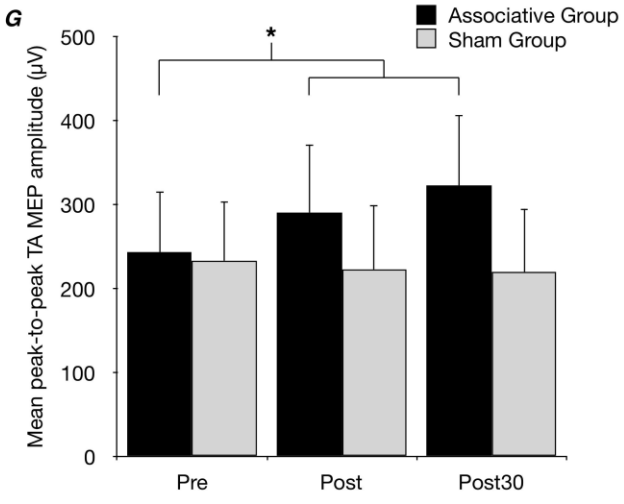


Table 1. Patient demographic data and baseline clinical evaluations.

Patient No.	Age, yr	Sex	Time After Stroke, days	Middle Cerebral Artery Side	Type of Lesion
			Associative Intervention Patient Group		
A01	57	M	63	L	Left media
A02	56	M	23	L	Left media
A03	58	M	18	R	Right basal ganglia
A04	66	M	44	L	Left basal ganglia
A05	54	F	108	R	Right basal ganglia
A06	66	M	37	R	Right basal ganglia
A07	64	M	16	L	Left corona radiata/media
A08	65	F	28	R	Right media
A09	64	M	67	R	Right corona radiata/media
A10	72	M	59	R	Right anterior cerebral artery
A11	42	M	103	L	Left basal ganglia
A12	66	M	54	L	Left media
Mean	**60.8**		**51.7**		
SD	**7.9**		**30.6**		
			Sham Intervention Patient Group		
S01	64	M	99	L	Left basal ganglia
S02	49	M	16	R	Right caps interna
S03	69	F	20	L	Left caps interna
S04	56	M	48	L	Left caps interna
S05	64	F	46	L	Left parito-occipt
S06	60	M	128	R	Right basal ganglia
S07	52	M	28	R	Right media
S08	77	M	57	L	Left basal ganglia
S09	54	M	59	L	Left basal ganglia
S10	53	M	22	L	Left media
S11	71	F	91	L	Left basal ganglia
S12	69	F	63	R	Right media
Mean	**61.5**		**56.4**		
SD	**8.9**		**34.8**		

M, male; F, female; R, right; L, left.

Table 2. Patient clinical and behavioral data.

Patient No.	LE-FM		mRS		FAC		ASS		10-m Walk Test, m/s	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Associative Intervention Patient Group										
A01	24	33	3	2	5	5	1	0	1.30	1.81
A02	27	34	2	2	4	5	0	0	1.85	2.04
A03	25	32	2	2	5	5	0	0	1.82	1.89
A04	28	32	4	2	3	4	0	0	1.13	1.69
A05	16	17	4	4	1	1	0	0	0.00	0.00
A06	18	31	4	4	0	1	0	0	0.00	0.00
A07	19	27	4	2	2	5	0	0	0.00	0.91
A08	31	32	4	3	2	4	0	0	0.00	0.91
A09	26	32	4	2	2	4	0	0	0.00	0.37
A10	10	12	4	4	1	3	0	0	0.00	0.31
A11	20	24	3	3	4	5	0	0	1.03	1.20
A12	23	32	4	3	2	4	0	0	0.00	1.91
Median	**23.5**	**32.0**	**4.0**	**2.5**	**2.0**	**4.0**	**0.0**	**0.0**		
IQR	**18.8-26.3**	**26.3-32.0**	**3.0-4.0**	**2.0-3.3**	**1.8-4.0**	**3.8-5.0**	**0.0-0.0**	**0.0-0.0**		
Mean									**0.59**	**1.09**
SD									**0.77**	**0.78**
Sham Intervention Patient Group										
S01	18	17	4	3	2	4	0	0	0.35	0.39
S02	28	31	3	2	4	5	0	0	1.51	1.51
S03	30	30	4	2	2	4	0	0	0.00	1.10
S04	14	21	4	4	1	1	0	0	0.00	0.00
S05	23	31	4	3	3	4	0	0	1.14	1.46
S06	18	21	4	4	0	1	0	0	0.00	0.00
S07	30	29	2	3	4	5	0	0	0.80	1.05
S08	22	25	4	2	2	4	0	0	0.00	0.60
S09	28	31	2	2	5	5	0	0	1.64	2.07
S10	33	34	2	2	5	5	0	0	1.56	2.19
S11	30	33	4	3	2	4	0	0	0.56	0.78
S12	15	18	4	3	1	4	1	0	0.00	0.20
Median	**25.5**	**29.5**	**4.0**	**3.0**	**2.0**	**4.0**	**0.0**	**0.0**		
IQR	**18.0-30.0**	**21.0-31.0**	**2.8-4.0**	**2.0-3.0**	**1.8-4.0**	**4.0-5.0**	**0.0-0.0**	**0.0-0.0**		
Mean									**0.63**	**0.95**
SD									**0.67**	**0.75**

mRS, modified Rankin scale score; FAC, functional ambulation classification; LE-FM, lower-extremity Fugl-Meyer assessment - motor performance; ASS, Ashworth scale for spasticity of the affected leg; IQR, interquartile range; SD, standard deviation.